UNITED STATES PATENT AND TRADEMARK OFFICE

UNITED STATES DEPARTMENT OF COMMERCE
United States Patent and Trademark Office
Address: COMMISSIONER FOR PATENTS
P.O. Box 1450
Alexandria, Virginia 22313-1450
www.uspto.gov



| APPLICATION NO. | FILING DATE | FIRST NAMED INVENTOR | ATTORNEY DOCKET NO. | CONFIRMATION NO. |
|---|---|---|---|---|
| 10/612,832 | 07/01/2003 | Harald Stein | 086035-000000US | 3864 |

20350 7590 05/21/2007

TOWNSEND AND TOWNSEND AND CREW, LLP
TWO EMBARCADERO CENTER
EIGHTH FLOOR
SAN FRANCISCO, CA 94111-3834

| EXAMINER | |
|---|---|
| YAO, LEI | |

| ART UNIT | PAPER NUMBER |
|---|---|
| 1642 | |

| MAIL DATE | DELIVERY MODE |
|---|---|
| 05/21/2007 | PAPER |

**Please find below and/or attached an Office communication concerning this application or proceeding.**

The time period for reply, if any, is set in the attached communication.

| Office Action Summary | Application No. | Applicant(s) | |
|---|---|---|---|
| | 10/612,832 | STEIN ET AL. | |
| | Examiner | Art Unit | |
| | Lei Yao, Ph.D. | 1642 | |

*-- The MAILING DATE of this communication appears on the cover sheet with the correspondence address --*

**Period for Reply**

A SHORTENED STATUTORY PERIOD FOR REPLY IS SET TO EXPIRE 3 MONTH(S) OR THIRTY (30) DAYS, WHICHEVER IS LONGER, FROM THE MAILING DATE OF THIS COMMUNICATION.

- Extensions of time may be available under the provisions of 37 CFR 1.136(a). In no event, however, may a reply be timely filed after SIX (6) MONTHS from the mailing date of this communication.
- If NO period for reply is specified above, the maximum statutory period will apply and will expire SIX (6) MONTHS from the mailing date of this communication.
- Failure to reply within the set or extended period for reply will, by statute, cause the application to become ABANDONED (35 U.S.C. § 133). Any reply received by the Office later than three months after the mailing date of this communication, even if timely filed, may reduce any earned patent term adjustment. See 37 CFR 1.704(b).

**Status**

1)☒ Responsive to communication(s) filed on *27 February 2007*.

2a)☒ This action is **FINAL**. 2b)☐ This action is non-final.

3)☐ Since this application is in condition for allowance except for formal matters, prosecution as to the merits is closed in accordance with the practice under *Ex parte Quayle*, 1935 C.D. 11, 453 O.G. 213.

**Disposition of Claims**

4)☒ Claim(s) *7-14,18,19,21,22 and 24-33* is/are pending in the application.

4a) Of the above claim(s) *8,10,12-14,19,21,22,24-28 and 31-33* is/are withdrawn from consideration.

5)☒ Claim(s) *18* is/are allowed.

6)☒ Claim(s) *7,9,11,29 and 30* is/are rejected.

7)☐ Claim(s) _____ is/are objected to.

8)☐ Claim(s) _____ are subject to restriction and/or election requirement.

**Application Papers**

9)☒ The specification is objected to by the Examiner.

10)☐ The drawing(s) filed on _____ is/are: a)☐ accepted or b)☐ objected to by the Examiner.

Applicant may not request that any objection to the drawing(s) be held in abeyance. See 37 CFR 1.85(a).

Replacement drawing sheet(s) including the correction is required if the drawing(s) is objected to. See 37 CFR 1.121(d).

11)☐ The oath or declaration is objected to by the Examiner. Note the attached Office Action or form PTO-152.

**Priority under 35 U.S.C. § 119**

12)☒ Acknowledgment is made of a claim for foreign priority under 35 U.S.C. § 119(a)-(d) or (f).

a)☐ All b)☐ Some * c)☐ None of:

1.☒ Certified copies of the priority documents have been received.

2.☐ Certified copies of the priority documents have been received in Application No. _____.

3.☐ Copies of the certified copies of the priority documents have been received in this National Stage application from the International Bureau (PCT Rule 17.2(a)).

* See the attached detailed Office action for a list of the certified copies not received.

**Attachment(s)**

1) ☒ Notice of References Cited (PTO-892)
2) ☐ Notice of Draftsperson's Patent Drawing Review (PTO-948)
3) ☐ Information Disclosure Statement(s) (PTO/SB/08) Paper No(s)/Mail Date _____.
4) ☐ Interview Summary (PTO-413) Paper No(s)/Mail Date. _____.
5) ☐ Notice of Informal Patent Application
6) ☐ Other: _____.

***Response to Argument and Amendment***

The Amendment filed on 2/27/2007 in response to the previous Non-Final Office Action to RCE (11/17/2006) is acknowledged and has been entered.

Claims 1-6, 15-17, 20, 23 are cancelled. Claims 31-33 are added. Claims 7-14, 18, 19, 21, 22, and 24-33 are pending. Claims 8, 10, 12-14 and 19, 21, 22, 24-28 have been withdrawn for non-elected invention previously. Newly added claims 31-33 depending on the withdrawn claim 19 are also withdrawn from consideration currently. Thus, claims 7, 9, 11, 18, 29 and 30 are under the consideration.

**The following office action contains NEW GROUNDS of rejection.**

***Rejections/Objections Withdrawn***

1. Claim objections (objections 2, 3, and 4) are withdrawn in view of the amendments to or cancellation of the claims.

2. Rejection of claims 1, 6, 7, 9, 11, 15-18 and 29-30 under 35 U.S.C. 112, second paragraph is withdrawn in view of cancellation of base claim 1.

3. Rejection of claims 1, 6, 7, 9, 11, 15-18 and 29-30 under 35 U.S.C. 112, first paragraph, as failing to comply with the written description requirement is withdrawn in view of cancellation of and amended to the claims.

4. Rejeciton of claims 1, 6, 15-17, and 29-30 under 35 U.S.C. 102(b) as being anticipated by Lemke et al., is withdrawn in view of cancellation of and amendment to the claims. However, the amended claims are still anticipted by Lemke et al., (see new grouph rejection below).

5. Rejections of claims 1, 6, 15-17, and 29-30 under 35 U.S.C. 102(e) as being anticipated by Mohler et al., is withdrawn in view of cancellation of and amendment to the claims.

6. Rejection of claims 9 and 11 under 35 U.S.C. 103(a) as being unpatentable over Mohler et al., applied in the claim 1 above and further in view of Deonarain et al., is withdrawn in view of cancellation of claim 1, however, the claims are unpatentable over Lemke et al., in view of Deonarain et al., (see new grouph rejection below).

***Response to Arguments***

***Sequence Requirements***

SEQ ID No(s) remain required for the sequences in specification on pages 12 and 15 and amended specification on page 16.

Applicant stats amendment made on June 15, 2004 to insert the sequence SEQ ID NO: 1-12 on page 12 and 15 of the specification. In response to this argument, the Office carefully review the file history and note that applicant amended specification on Sep 09 2005, in which page 16 contain the sequence, which have no SEQ ID NOs associated. Thus, applicant needs to comply with the sequence rules to add SEQ ID NOs to those sequences and to check the entire disclosure and file history to ensure that the application is in sequence compliance.

Any questions regarding compliance with the sequence rules requirements specifically should be directed to the departments listed at the bottom of the Notice to Comply (see attached form, PTO L90).

***Claim Objections***

The claim 9 remain objected to as failing to provide proper antecedent basis for the claimed subject matter as the following because Applicant does not response to this objection or amend claim 9 based on the objection.

Claim 9 is dependent on claim 8, which is drawn to a non-elected invention (toxin). Morever, claim 8 is only drawn to a toxin, claim 9 is drawn to toxic proteins, enzymes, or proenzymes, claim 9 does not further limit the claim 8. For the purpose of examination, claim 9 is treated as further drawn to claim 1.

***The following is a New Ground of rejection-based on the amendment to the claims***

***Claim Rejections - 35 USC § 102***

The following is a quotation of the appropriate paragraphs of 35 U.S.C. 102 that form the basis for the rejections under this section made in this Office action:

A person shall be entitled to a patent unless –

> (b) the invention was patented or described in a printed publication in this or a foreign country or in public use or on sale in this country, more than one year prior to the date of application for patent in the United States.

Claims 7 and 29-30 are rejected under 35 U.S.C. 102(b) as being anticipated by Lemke et al., (US Patent NO: 6033876, Mar, 2000, provide in previous office action).

Amended claim 7 is drawn to a reagent that binds CD30 wherein the reagent is a humanized version of the antibody that binds CD30 at the same epitope as the antibody produced by the cell DSZ1 (claim 7(3)). Claims 29-30 are drawn to a composition or a CD30 diagnosing kit comprising the reagent of claim 7. For this rejection the intended use of a composition and diagnostic agent is given no patentable weight.

Lemke et al., disclose anti-CD30 antibody, Ber-H2, binding to CD30 epitope. Lemke et al., disclose that the antibody can be used as whole monoclonal antibodies and humanized antibody for treating a disease (col 4, line 52-60, and col 6). Ber-H2 antibody disclosed by Lemke et al., binds to the same epitope of CD30 shown as amino acid sequence DCRKQCEPDYYLD and GDCRKQCEPDYYL (see specification page 15) as evidenced by Dong et al., (figure 1, J of Molecular Recognition, vol 16 page 28-36, 2003). Lemke et al., also disclose a composition comprising the antibody to CD30 (col 6, line 20-30), which is used for inhibiting release of soluble CD30 (col 2 and col 9-10).

Although Lemke et al., do not explicitly teach a kit containing the antibody for diagnosing or treating CD30 related disease, claim 30 is anticipated by Lemke et al., because Lemke et al., disclose diagnosis of a disease with the antibodies to CD30, because formation of a kit using known component is within the purviews of one skilled in the art and because claim 30 recites a kit comprising antibody to CD30 for diagnosing a disease and a instruction of using the reagent. See MPEP 2112.01-III as following:

> Where the only difference between a prior art product and a claimed product is printed matter that is not functionally related to the product, the content of the printed matter will not distinguish the claimed product from the prior art. In re Ngai, ___ F.3d ___, 2004 WL 1068957 (Fed. Cir. May 13, 2004.

Response to applicant's argument.

Applicant argues the rejection (Lemke et al., page 11) and states in view of the present amendment "the structural feature of the CD30-binding reagent in its CD30-binding domain are the same as the antibody produced by the DSMACC2548 cell or contain only specifically defined modification" and states "amended claims is free of art". In responses to this argument, claim 7 as amended, is drawn to an antibody to CD30 that binds to the same epitope as the antibody produced by cell DSZ1. As stated in the rejection above, Lemke et al. disclose antiCD30 antibody, Ber-H2, that binds to the same epitope as the antibody produced by cell DSZ1. Thus, Applicant's argument has not been found persuasive, and the rejection is made for reason of the record.

***Claim Rejections - 35 USC § 103***

The following is a quotation of 35 U.S.C. 103(a) which forms the basis for all obviousness rejections set forth in this Office action:

> (a) A patent may not be obtained though the invention is not identically disclosed or described as set forth in section 102 of this title, if the differences between the subject matter sought to be patented and the prior art are such that the subject matter as a whole would have been obvious at the time the invention was made to a person having ordinary skill in the art to which said subject matter pertains. Patentability shall not be negatived by the manner in which the invention was made.

Claims 9 and 11 remain rejected under 35 U.S.C. 103(a) as being unpatentable over Lemke et al., and evidenced by Dong et al., applied in the claim 7 above and further in view of Deonarain et al., (Br J Cancer, vol 70 page 786-94, 1994, provided in previous office action).

Claims 9 and 11 are further drawn to claim 7 (see objection), wherein the reagent is linked with enzymes from the group of the phosphodiesterases.

Lemke et al., teach humanized anti-CD30 epitope antibody, Ber-H2.

Lemke et al., do not teach that the antibody is linked to an enzymes or phosphodiesterases.

Deonarain et al., teach ribonuclease (RNas), an enzyme from group of phosphodiesterases, and using the enzyme for cancer therapy. Deonarain et al., also teach that RNase is fused to an antibody or an antibody fragment. Deonarain et al., further teach that the fusion protein is cytotoxic to the cells at low concentrations (page 792, column 1, paragraph 1).

One of ordinary skill in the art at the time the invention was made would have been motivated to combine the teaching of Deonarain et al., with the teaching of Lemke et al., in order to benefit the conjugated antibody for treating a disease associated with CD30 antigen expression comprising cancer because Deonarain et al., teach that RNase fused to an antibody is used for the cancer therapy. One of ordinary skill in the art at the time the invention was made would have had a reasonable expectation of success to make the conjugated antibody linked to a phosphodiesterases by combining the teaching of Deonarain et al., with the teaching of Lemke et al., because Deonarain et al., have shown the enzyme and a method of making the conjugate antibody comprising the enzyme and Lemke et al., have shown the Ber-H2, binding to the same epitope of CD30 as antibody produced by cell DSZ1 evidenced by Dong et al. Therefore, the invention as a whole would have been prima facie obvious to one of ordinary skill in the art at the time the invention was made, absent unexpected results.

Response to applicant's argument.

Applicant argues the rejection (Lemke et al., in view of Deonarain, page 11) for the same reason, which has been discussed above. Thus, Applicant's argument has not been found persuasive, and the rejection is made for reason of the record.

***Conclusion***

Claim 18 is allowed

Applicant's amendment necessitated the new ground(s) of rejection presented in this Office action. Accordingly, **THIS ACTION IS MADE FINAL.** See MPEP § 706.07(a). Applicant is reminded of the extension of time policy as set forth in 37 CFR 1.136(a).

A shortened statutory period for reply to this final action is set to expire THREE MONTHS from the mailing date of this action. In the event a first reply is filed within TWO MONTHS of the mailing date of this final action and the advisory action is not mailed until after the end of the THREE-MONTH shortened statutory period, then the shortened statutory period will expire on the date the advisory action is mailed, and any extension fee pursuant to 37 CFR 1.136(a) will be calculated from the mailing date of the advisory action. In no event, however, will the statutory period for reply expire later than SIX MONTHS from the date of this final action.

Any inquiry concerning this communication or earlier communications from the examiner should be directed to Lei Yao, Ph.D. whose telephone number is 571-272-3112. The examiner can normally be reached on 8am-6.00pm Monday-Thursday.

Any inquiry of a general nature, matching or file papers or relating to the status of this application or proceeding should be directed to Kim Downing for Art Unit 1642 whose telephone number is 571-272-0521

If attempts to reach the examiner by telephone are unsuccessful, the examiner's supervisor, Shanon Foley can be reached on 571-272-0898. The fax phone number for the organization where this application or proceeding is assigned is 571-273-8300.

Information regarding the status of an application may be obtained from the Patent Application Information Retrieval (PAIR) system. Status information for published applications may be obtained from either Private PAIR or Public PAIR. Status information for unpublished applications is available through Private PAIR only. For more information about the PAIR system, see http://pair-direct.uspto.gov. Should you have questions on access to the Private PAIR system, contact the Electronic Business Center (EBC) at 866-217-9197 (toll-free).

Lei Yao,
Examiner
Art Unit 1642

LY

SHANON FOLEY
SUPERVISORY PATENT EXAMINER
TECHNOLOGY CENTER 1600

| Notice of References Cited | Application/Control No. 10/612,832 | Applicant(s)/Patent Under Reexamination STEIN ET AL. | |
|---|---|---|---|
| | Examiner Lei Yao, Ph.D. | Art Unit 1642 | Page 1 of 1 |

**U.S. PATENT DOCUMENTS**

| * | | Document Number Country Code-Number-Kind Code | Date MM-YYYY | Name | Classification |
|---|---|---|---|---|---|
| | A | US- | | | |
| | B | US- | | | |
| | C | US- | | | |
| | D | US- | | | |
| | E | US- | | | |
| | F | US- | | | |
| | G | US- | | | |
| | H | US- | | | |
| | I | US- | | | |
| | J | US- | | | |
| | K | US- | | | |
| | L | US- | | | |
| | M | US- | | | |

**FOREIGN PATENT DOCUMENTS**

| * | | Document Number Country Code-Number-Kind Code | Date MM-YYYY | Country | Name | Classification |
|---|---|---|---|---|---|---|
| | N | | | | | |
| | O | | | | | |
| | P | | | | | |
| | Q | | | | | |
| | R | | | | | |
| | S | | | | | |
| | T | | | | | |

**NON-PATENT DOCUMENTS**

| * | | Include as applicable: Author, Title Date, Publisher, Edition or Volume, Pertinent Pages) |
|---|---|---|
| | U | Dong et al., J of Molecular Recognition, vol 16 page 28-36, 2003. |
| | V | |
| | W | |
| | X | |

*A copy of this reference is not being furnished with this Office action. (See MPEP § 707.05(a).)
Dates in MM-YYYY format are publication dates. Classifications may be US or foreign.

JOURNAL OF MOLECULAR RECOGNITION
*J. Mol. Recognit.* 2003; **16**: 28–36
Published online in Wiley InterScience (www.interscience.wiley.com). **DOI**:10.1002/jmr.605

# Human CD30: Structural implications from epitope mapping and modeling studies

Liying Dong[1]†, Martin Hülsmeyer[2]‡, Horst Dürkop[3], Hinrich P. Hansen[4], Jens Schneider-Mergener[1], Andreas Ziegler[2]* and Barbara Uchanska-Ziegler[2]

[1]Institut für Medizinische Immunologie, Universitätsklinikum Charité, Humboldt-Universität zu Berlin, Berlin, Germany
[2]Institut für Immungenetik, Universitätsklinikum Charité, Humboldt-Universität zu Berlin, Berlin, Germany
[3]Institut für Pathologie, Universitätsklinikum Benjamin Franklin, Freie Universität Berlin, Berlin, Germany
[4]Department of Medicine I, Universität Köln, Germany

**The human CD30 molecule is expressed transiently at very low levels on intrafollicular and perifollicular T and B cell blasts in lymphoid tissues, but is specifically upregulated on certain tumor cells, e.g. Hodgkin and Reed–Sternberg (H-RS) cells. With its specific expression pattern and easy accessibility on the surface of H-RS cells CD30 is a valuable diagnostic marker and holds considerable promise as a target for *in vivo* immunotherapy. Knowledge of epitopes on the CD30 molecule is expected to facilitate the design of novel non-immunogenic anti-CD30 reagents. Therefore, we have mapped the epitopes of several monoclonal antibodies (mAb) applying a peptide array of overlapping CD30-derived peptides. For the mAb Ber-H2, two linear epitopes with identical sequence were found, while the mAb Ki-2 and the single chain Fv fragment R4-4 each recognized a single linear antigenic determinant, respectively. On the other hand, the mAb Ki-1 bound to a discontinuous epitope composed of two regions, one located near the N-terminus and the other near the membrane-spanning region of CD30. Using molecular modeling, it was possible to visualize the location of the epitopes on exposed loop regions of the molecule within the N-terminal domain. Finally, the results obtained with the mAb Ki-1 imply that the ends of the N- and C-terminal parts of the extracellular portion of CD30 are in close vicinity of each other, suggesting a flower-like structure for the membrane-bound homotrimeric CD30 molecule. Copyright © 2003 John Wiley & Sons, Ltd.**

*Keywords:* **CD30; monoclonal antibody; epitope mapping; peptide array; homology modeling**

*Received 24 October 2001; revised 30 September 2002; accepted 16 October 2002*

## INTRODUCTION

CD30 was originally described as a human cell surface molecule recognized by the Ki-1 monoclonal antibody (mAb) (Schwab *et al.*, 1982). Under physiological conditions, CD30 is transiently expressed at very low levels on intrafollicular and perifollicular T and B cell blasts in lymphoid tissues. It is specifically upregulated on certain tumor cells, e.g. Hodgkin and Reed–Sternberg cells, or in anaplastic large cell lymphomas (ALCL), in diffuse large B cell lymphomas of the anaplastic variant, in adult T cell leukaemia (Stein *et al.*, 1985), and in embryonal carcinoma of the testis (Pallesen and Hamilton-Dutoit, 1988). The highly restricted expression pattern of the CD30 molecule, that has recently been attributed to the regulation by a minimal promoter (Dürkop *et al.*, 2000), makes CD30 a very useful target for diagnostic and therapeutic applications, although the function of this protein is not fully understood (Muta *et al.*, 2000; Amakawa *et al.*, 1996; Horie and Watanabe, 1998).

Human CD30 is a glycosylated type I transmembrane receptor. Its 577 amino acid residues can be subdivided into an extracellular part with 361 residues and a cytoplasmatic part with 188 residues. The ectodomain is very cysteine-rich (34 cysteines), consists of two domains (d1 and d2) connected by a linker, and shows significant homology to other members of the tumor necrosis factor receptor (TNFR) superfamily (Dürkop *et al.*, 1992; Locksley *et al.*, 2001). The natural ligand for CD30 is CD153 (originally CD30L), a 233 residue type II glycoprotein, which is part of the TNF family (Armitage, 1997). Like other members of this family, CD153 forms homotrimers which are considered to be the functional form of this protein (Locksley *et al.*, 2001). Recently, a pre-ligand-binding assembly domain (PLAD) has been identified in the N-terminal domain of TNFR, and shown to be responsible for self-assembly even in the absence of ligand (Chan *et al.*, 2000). The normal functions of TNF/TNFR proteins depend on the obligatory 3-fold symmetry that defines the essential signaling stoichiometry (Locksley *et al.*, 2001). During our studies with the CD30 ectodomain (CD30[ex]), gel filtration experients revealed for the first time that also CD30 forms these homotrimeric

*Correspondence to: A. Ziegler, Institut für Immungenetik, Universitätsklinikum Charité, Humboldt-Universität zu Berlin, Spandauer Damm 130, 14050 Berlin, Germany.
E-mail: andreas.ziegler@charite.de
†Present address: Jerini AG, Invalidenstrasse 130, 10115 Berlin, Germany.
‡Present address: Institut für Kristallographie, Freie Universität Berlin, Germany.
*Contract/grant sponsor:* Deutsche Forschungsgemeinschaft; *contract/grant number:* SFB449, TPB5.

**Abbreviations used:** CD30[ex], CD30 extracellular domain; CRD, cysteine-rich domain; mAb, monoclonal antibody; scFv, single chain Fv fragment; TNFR, tumor necrosis factor receptor; wt, wild-type.

**Table 1. Alanine mutagenesis analysis**

| | Abbreviation | wt sequence | Variant produced |
|---|---|---|---|
| Ber-H2 epitope 1 | E1 | $^{49}$KQCEPDYYL$^{57}$ | $^{49}$KQCAPAYYL$^{57}$ |
| Ber-H2 epitope 2 | E1′ | $^{224}$KQCEPDYYL$^{232}$ | $^{224}$KQCAPAYYL$^{232}$ |
| Ber-H2 (epitopes 1 + 2) | E1 + E1′ | Combination of E1 and E1′ | KQCAPAYYL (2×) |
| Ki-1 epitope 1 | E4α | $^{9}$DTCHGNP$^{15}$ | $^{9}$DTCAGAP$^{15}$ |
| Ki-1 epitope 2 | E4ω | $^{346}$LPIPTSAP$^{353}$ | $^{346}$APAPTSAP$^{353}$ |

complexes *in vitro* (unpublished results). Currently, no detailed structural information on the CD30 molecule or its ligand CD153 is available.

In addition to Ki-1, several murine mAb recognizing human CD30 molecules have been generated (Lotze and Zola, 1997), of which Ber-H2 and HRS-3 have already been employed for both the diagnosis and the treatment of CD30-positive malignancies (Falini *et al.*, 1992; Hartmann *et al.*, 1997). Without exception, all these reagents recognize the extracellular part of the molecule (Lotze and Zola, 1997). Their epitopes are unknown, since only competition studies among some of these mAb as well as with CD153 have been carried out (Horn-Lohrens *et al.*, 1995; Franke *et al.*, 2000). However, the epitopes of Ki-1 and Ber-H2 have already been mapped roughly to the N-terminal half of CD30[ex] (Dürkop *et al.*, 1992). Knowledge of antibody epitopes on the CD30 molecule is a prerequisite for the design of CD30-specific reagents other than antibodies, e.g. RNA-aptamers (Nolte *et al.*, 1996; Klussmann *et al.*, 1996). Such reagents could overcome problems associated with the application of immunogenic murine antibodies during *in vivo* therapy.

Here we report on the identification of binding sites for the mAb Ki-1, Ki-2, Ber-H2 and a human CD30-specific single chain Fv (scFv) fragment termed R4-4 using a peptide array of tridecameric, overlapping CD30-derived peptides prepared by spot synthesis (Frank, 1992). Using molecular modeling, we were able to identify the epitopes as accessible loop regions within the N-terminal region of the extracellular domain. The results have unexpected implications for the three-dimensional structure of the molecule.

## MATERIALS AND METHODS

### Antibodies

The mAb Ber-H2, Ki-1, and Ki-2 were employed as culture supernatant or purified protein (Schwab *et al.*, 1982; Schwarting *et al.*, 1989; Horn-Lohrens *et al.*, 1995). The scFv R4-4 was obtained by selecting a human phage display library ('Tomlinson I') on purified CD30[ex] molecules (Hemmann *et al.*, 2002). mAb or scFv of irrelevant reactivity were employed as negative controls.

### Peptide synthesis

Peptides were produced at 50 nmol/cm$^2$ on Whatman 50 cellulose membranes (Whatman, Maidstone, UK) using an Abimed spot synthesizer (Langenfeld, Germany). The method has been described in detail previously (Frank, 1992; Kramer and Schneider-Mergener, 1998). For the peptide scans, the whole extracellular part of CD30 was synthesized as a set of 117 overlapping tridecameric peptides. For substitutional analyses, peptides showing interaction with an antibody were systematically replaced by all 20 L-amino acids at each position and tested for antibody binding. The software LISA (Jerini AG, Berlin, Germany) was used to generate the sequence files.

### Identification of epitopes

Membranes with covalently bound peptides were blocked for 4 h with blocking buffer consisting of 10% blocking reagent (Boehringer Mannheim, Mannheim, Germany) in T-TBS (Tris-buffed saline 50 mM, pH 8.0/0.05% Tween®-20 containing 1% sucrose). The membranes were washed for 10 min in T-TBS. Subsequently, they were incubated overnight at 4 °C with cell culture supernatant containing unconjugated Ber-H2, Ki-1, Ki-2 (1–2 μg/ml each) or R4-4 (4 μg/ml), respectively. Thereafter, the membranes were washed three times for 5–10 min with T-TBS, followed by a 1 h incubation with a peroxidase-labeled goat anti-mouse IgG antibody (Sigma, Munich, Germany, 1–2 μg/ml in blocking buffer) in the case of Ber-H2 or Ki-2. Instead, in the case of Ki-1, EnVision™ (diluted 1:200 in blocking buffer) (DAKO Diagnostika, Hamburg, Germany) was used according to manufacturer's recommendations, while a peroxidase-labeled goat anti-human IgG (F(ab')$_2$ specific) at 1 μg/ml (Jackson ImmunoResearch, West Grove, PA, USA) was used for scFv R4-4. All membranes were also incubated with secondary antibodies alone as control. The detection was accomplished with the SuperSignal chemiluminescence detection system (Pierce, Rockford, IL, USA) and the Lumi-Imager™ (Boehringer Mannheim, Germany). Data acquisition and evaluation were performed with the software LumiAnalyst™ (Boehringer Mannheim, Germany).

### Alanine mutagenesis

Recombinant CD30 cDNA in plasmid pMT/BiP/V5-His A (Invitrogen) was used for the expression of the extracellular portion of the CD30 protein (CD30[ex]) fused to a C-terminal 6x-His-tag. The generation of this construct will be described elsewhere (Hemmann *et al.*, in preparation). Mutations were introduced by the mutagenesis kit Quikchange (Stratagene) following the manufacturer's instructions. The following primers were used to obtain the variants described in Table 1: E4α: 5′-CTTCGAGGA-

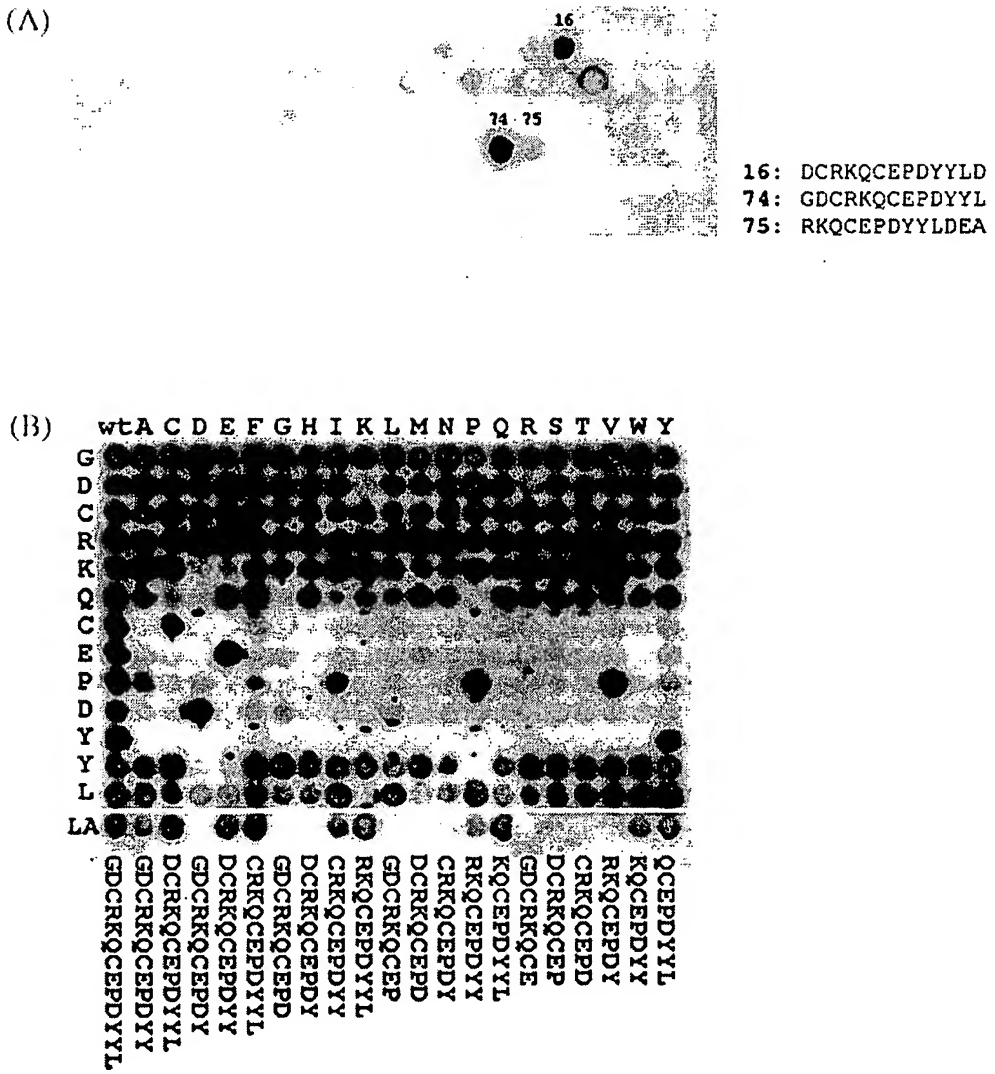


**Figure 1.** Epitope analysis of Ber-H2. (A) Epitope mapping employing the complete set of CD30-derived overlapping tridecameric peptides (shifted by three amino acids) from the extracellular part of the CD30 molecule (361 amino acids) prepared by SPOT synthesis after probing with Ber-H2. The first spot comprises residues 1–13 of the CD30 ectodomain, the second residues 4–17 etc., yielding 117 spots in total. (B) Substitutional analysis of the Ber-H2 epitope. Each single residue of the starting peptide (peptide 74 from A, left column, identical peptides as control) was substituted by all 20 L-amino acids (rows) and the resulting peptides were analyzed for binding by Ber-H2. In the last row (LA), the Ber-H2 binding peptide was shortened stepwise at both termini (peptide sequences are shown under the spots) to determine the length of the epitope.

CACCTGTGCTGGAGCCCCCAGCCACTAC-3′ and reverse complement; E4$\omega$: 5′-GCCAGTAAGACGGCGCCCGCCCCAACCAGCGCTC-3′ and reverse complement; E1: 5′-GACTGACTGCAGGAAGCAGTGTGCGCCTGCCTACTACCTG-3′ and reverse complement; E1′: 5′-TGGTGATTGCAGGAAGCAGTGTGCGCCTGCCTACTACCTG-3′ and reverse complement. Variant CD30 [ex]E1 + E1′ was constructed from CD30[ex]E1 using the same E1′ primer pair as before for the generation of CD30[ex]E1′. The constructs were transformed into *E. coli* XL Blue. Recombinant plasmids were isolated with Nucleobond AX columns (Macherey & Nagel, Düren, Germany), the concentration determined at 260 nm and the insert sequenced for integrity.

### Transfection of Drosophila S2 cells

Purified constructs were transiently transfected to *Drosophila* Schneider S2 cells (Invitrogen) according to the manufacturer's recommendations. Three days after transfection, the cells were induced with 500 μM copper sulfate. The supernatant was harvested after 5 days of induction and used for experiments without further manipulation.

### Analysis of recombinant CD30ex in SDS–PAGE and western blots

Culture supernatant was run under non-reducing conditions

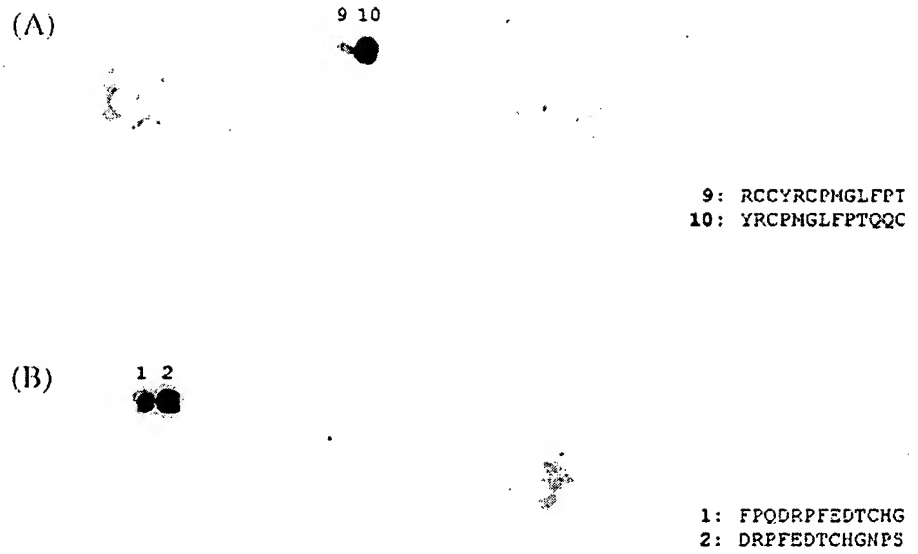


**Figure 2.** Epitope mapping of Ki-2 and R4-4. Ki-2 (A) and R4-4 (B) antibody epitopes were identified applying identical spot membranes as described in Fig. 1(A).

in 12% SDS–polyacrylamide gels. Gels were stained with Coomassie Brillant blue R-250 or blotted onto nitrocellulose membranes (Schleicher & Schuell). Blots were blocked with 2% skimmed milk powder, 2% Tween-20 in PBS pH 7.4 and incubated with the mAb Ber-H2 (64 ng/ml) and Ki-1 (360 ng/ml) followed by incubation with goat anti-mouse IgG-HRP (DAKO, 100 ng/ml). PBS with pH 7.4 was used for all washing steps. The binding of Ber-H2 or Ki-1 to CD30[ex] was visualized by use of OptiMist-HRP (Intergen).

### BIAcore analyses

Surface plasmon resonance (SPR) measurements were carried out using the BIAcoreX system (Biacore AB Uppsala, Sweden). For analysis of antibody–peptide interactions, the mAb Ki-1 was coupled to CM5 sensor chips using the EDC/NHS kit, yielding approximately 6500 RU. For the reference chip, an anti-p24 HIV-1 mAb was used. Interaction analysis was performed in 10 mM Hepes, 150 mM NaCl, 3.4 mM EDTA and 0.005% surfactant P20 pH 7.4 (HBS) at 25 °C with a 15 µl/min flow rate. $K_D$ values could be determined by applying the steady-state model with the BIAevaluation 3.1 software. Binding of CD30-derived peptides FEDTCHGNPSHYY (peptide 1, bearing the Ki-1 E4$\alpha$-epitope, spot 3) and LPIPTSAPVALSS (peptide 2, bearing the Ki-1 E4$\omega$-epitope, spot 116) to Ki-1 was measured at five different concentrations (25–400 µM).

### Homology modeling

The first step of the homology modeling was performed using the automated knowledge-based protein modeling server SwissModel at Expasy (Peitsch, 1996). The structure of TNFR1 (PDB-code 1ext; Naismith *et al.*, 1996) was defined as target model. The resulting model structure only contained the N-terminal domain (residues 19–144) of CD30, because TNFR1 has a much shorter ectodomain than CD30[ex].

Sequence alignments of proteins of the TNFR superfamily show conserved patterns of disulfide bridges. As the novel model did not contain all disulfide bridges that could be expected, two missing bridges were introduced manually, using the graphics package O (Jones and Kjeldgaard, 1997). Afterwards, polar hydrogen atoms were added and their position relaxed by 500 steps of steepest descent minimization (Insight, MSI, San Diego, CA, USA). Finally, the whole protein was minimized by 1000 steps of conjugate gradient minimization. Inspection of the stereochemical properties of the model showed all parameters in the allowed regions.

## RESULTS

The epitopes of the mAb Ber-H2, Ki-1, and Ki-2 as well as that of the scFv fragment R4-4 were determined by assessing their reactivity with overlapping tridecameric CD30 peptides spotted onto cellulose membranes.

### mAb Ber-H2

The mAb Ber-H2 showed two strong binding signals [Fig. 1(A)]: peptide 16 (with the sequence $^{45}$DCRKQCEPDYYLD$^{57}$) and peptide 74 ($^{219}$GDCRKQCEPDYYL$^{231}$). A much weaker signal was visible for peptide 75 ($^{222}$RKQCEPDYYLDEA$^{234}$). Peptides 16 and 74 had nearly identical sequences, while peptide 75 overlapped with peptide 74 by 10 amino acids. A substitutional analysis of the peptide 74 sequence [Fig. 1(B)], replacing each single residue of the epitope by all 20 L-amino acids, revealed key residues for interaction. The core sequence of the epitope was CEPDY, which was found to be almost invariable with the exception of the proline, that could be substituted by A, I or V although with reduction of Ber-H2 binding activity for the former two residues. Two additional residues that flank the core epitope, were also important for Ber-H2 binding, but showed a higher variability: KQ and YL. Sequential N- or C-terminal shortening of the peptide 74 [Fig. 1B, last row] supported

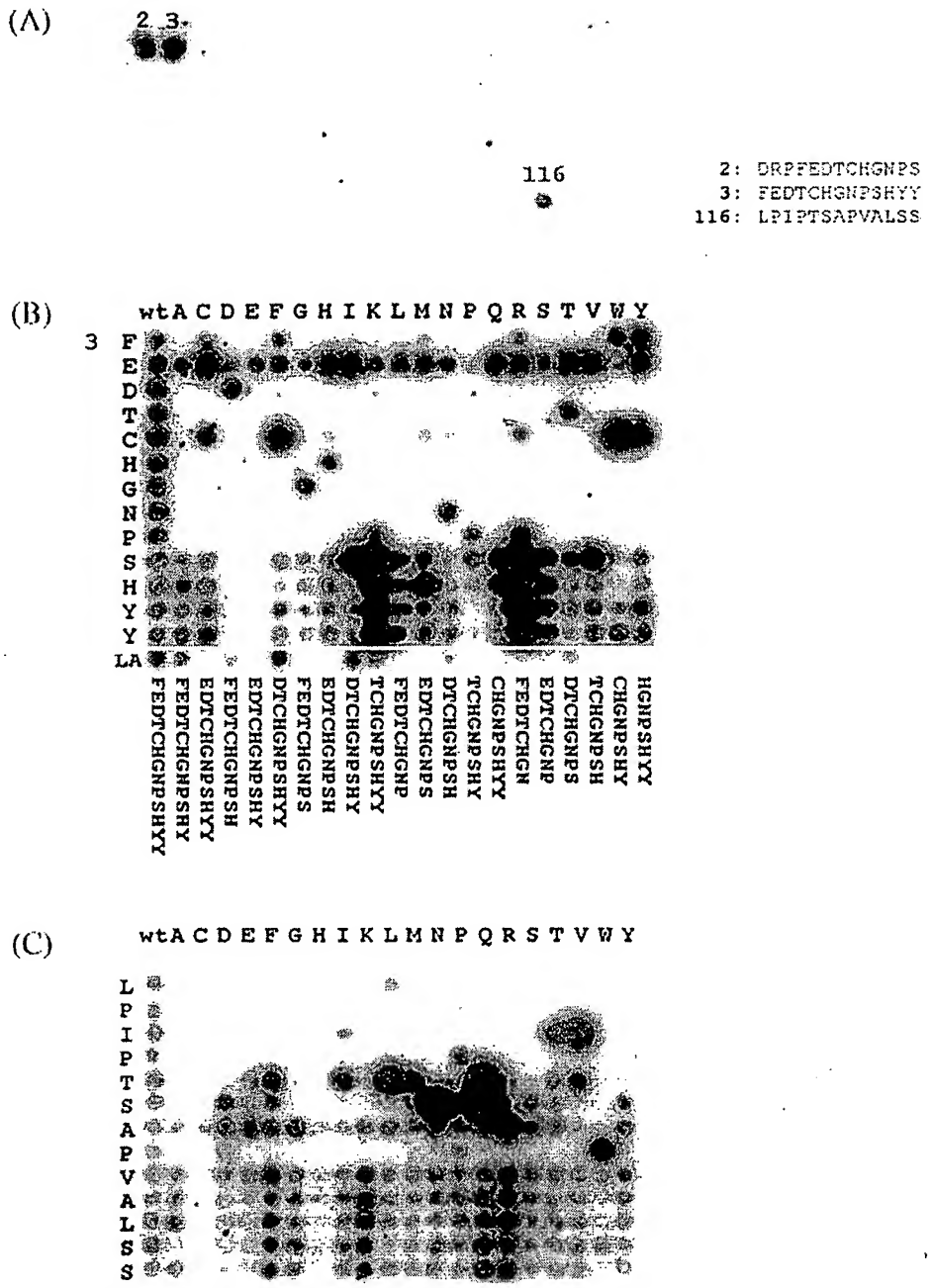


**Figure 3.** Epitope analysis of Ki-1. (A) The Ki-1 epitope was identified according to Fig. 1(A). (B) Substitutional analysis of the Ki-1 epitope E4$\alpha$ [peptide 3 from A, for further explanations see Fig. 1(B)]. (C) Substitutional analysis of the Ki-1 epitope E4$\omega$ (peptide 116 from A).

this notion. Furthermore, at least one of the preceding residues seems to be important for recognition by Ber-H2 as well, because peptide 17 (which lacks the N-terminal DCR of peptide 16) was unreactive. Obviously, Ber-H2 had two possibilities to interact with CD30: one epitope (termed E1) was located in the first third of the N-terminal domain, the second (E1′) at a comparable position in the C-terminal domain of the extracellular part of the molecule.

### mAb Ki-2 and scFv R4-4

Like Ber-H2, Ki-2 clearly recognized a linear epitope (E2) as well (peptides 9 and 10), located between the first Ber-H2 and the R4-4 epitopes [see below, Fig. 2(A)]. The common residues of these peptides corresponded to the sequence YRCPMGLFPT. However, it was evident from the intensity of the peptide reactivities that the last three residues in the peptide 10 were beneficial for recognition by Ki-2. The N-terminal YRC motif seemed to be indispensable for Ki-2 recognition as peptide 11, lacking these residues, showed no reactivity. A substitutional analysis was not performed.

Incubation of the scFv R4-4 with the peptide array gave reactivity with peptides 1 and 2, in immediate vicinity of the CD30 N-terminus [Fig. 2(B)]. Furthermore, replacement analysis similar to that described for Ber-H2 revealed that the

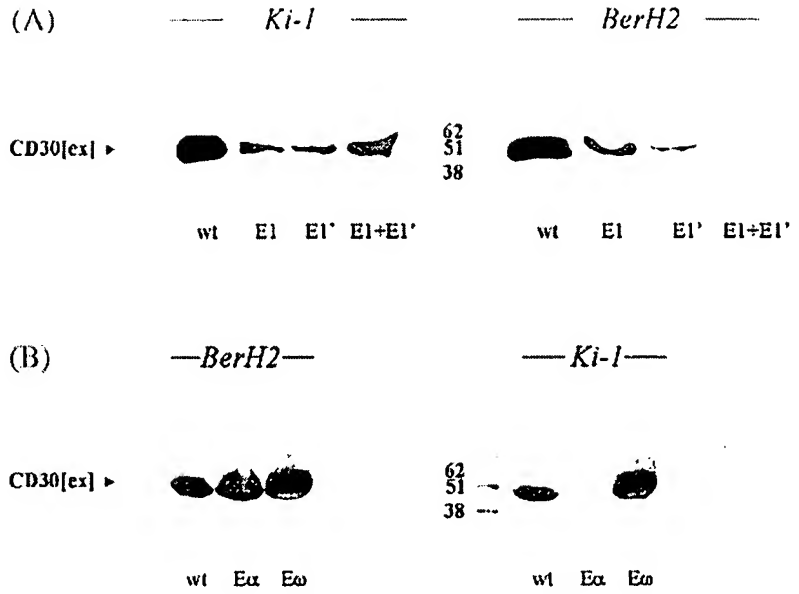


**Figure 4.** Western blot analysis of the Ber-H2 and Ki-1 epitope mutants. (A) Western blots of supernatants of S2 cells transiently transfected with CD30[ex] Ber-H2 epitope mutants. In the left panel Ki-1 was used as first Ab, in the right panel Ber-H2. In both cases goat anti-mouse EnVision was used as second Ab. (B) Western blots of supernatants of S2 cells transiently transfected with CD30[ex] Ki-1 epitope mutants. In the left panel Ber-H2 was used as first Ab, in the right panel Ki-1. In both cases goat anti-mouse EnVision was used as second Ab.

R4-4 epitope was RPFXDXC (data not shown), explaining why peptide 3 was unreactive. The R4-4 epitope (E3) did not overlap with the Ber-H2 or Ki-2 binding regions, but overlapped with one of the Ki-1 epitopes (see below).

### mAb Ki-1

Reaction of the peptide array with Ki-1 led to two strongly binding peptides (2, 3) and one weakly binding peptide [116, Fig. 3(A)]. Peptides 2 and 3 were located near the N-terminus of the molecule, whereas peptide 116 was found at the C-terminal end of CD30[ex], close to the transmembrane domain. This result pointed to the existence of a conformational determinant for Ki-1, quite in contrast to the continuous epitopes observed for Ber-H2, Ki-2, and R4-4, but supported by amino acid replacement analysis of peptides 3 and 116 [Fig. 3(B, C)]. The core epitope of Ki-1 was found to be DTCHGNP, close to the N-terminus, as almost every substitution in this region resulted in the loss of recognition. The only residue that allowed limited variability was the cysteine, as it could be replaced by large aromatic residues (F, Y, W). In order to clearly identify the borders of this first part of the Ki-1 epitope, the tridecameric peptide 3 was gradually shortened [Fig. 3(B), last row]. The peptide DTCHGNPS was recognized very weakly, but DTCHGNPSHY was recognized well, showing that additional residues flanking the core epitope at its C-terminus were favoring recognition, although the nature of these residues was relatively unimportant as visible from the substitution analysis. Similar considerations indicated that amino acids at the peptide N-terminus (FE) may also play a comparable stabilizing role, although they were not absolutely essential for recognition. The second part of the discontinuous Ki-1 epitope (peptide 116) showed a much weaker signal. From the replacement analysis, the sequence LPIPXXXP was identified [Fig. 3(C)]. Again, the first four and the last residue showed little tolerance with regard to replacements. Isoleucine could be replaced by T or V, and the C-terminal proline of the core epitope by W.

From our past observations, the drastic intensification of the reactivity of some peptides upon mutation (e.g. peptide3, K and R variants) is an effect that typically occurs in discontinuous epitopes (Reineke *et al.*, 1999a). In the case of a continuous epitope an interacting antibody paratope has an optimized shape for binding and any mutation in the epitope can only lead to very minor increase or a clear loss of affinity (see e.g. the Ber-H2 epitopes, for review see Reineke *et al.*, 1999b). The situation is different envisaging a discontinuous epitope: as the peptides studied for binding represent only a part of the entire epitope, mutations of nonessential residues can lead to enhanced binding. Again, the paratope is optimized for the complete epitope, consequently the two parts cannot evolve independently. If they are separated and examined *in vitro*, epitopes with better binding than the wild-type (wt) can be generated, as the second part of the epitope is missing.

### Alanine mutagenesis

To verify the correctness of the identified Ber-H2 and Ki-1 epitopes, mutational studies were undertaken to prove that these epitopes were also recognized in the context of the

intact protein. For this purpose, double alanine mutants of the epitopes were created. Prolines, cysteines and glycines were left unmodified as they often play important roles for the tertiary structure of a protein. Additionally, only those residues were selected, that revealed a clear loss of activity when mutated to alanine as shown by the mutational analysis of the respective peptide (see above). Introducing two of these mutations should abrogate antibody binding and consequently confirm the epitope. Table 1 lists the mutations introduced to CD30[ex]. The CD30[ex] constructs were transiently transfected into *Drosophila* S2 cells, and the culture supernatants containing the variants were then analyzed by western blotting with Ki-1 and Ber-H2.

The western blot of the Ber-H2 epitope mutants [Fig. 4(A)] was in agreement with the expectations: CD30[ex] and all the mutated forms were detected with Ki-1. However, Ber-H2 recognized the wt and the single epitope mutants (E1 or E1'), but no signal could be observed when both epitopes were mutated (E1 + E1'). This proved that the Ber-H2 epitopes had been correctly identified by the spotted peptide library approach.

The analysis of the Ki-1 epitope mutants [Fig. 4(B)] gave a different result. wt-CD30[ex] was recognized by both Ki-1 and Ber-H2. As expected, Ber-H2 also detected both variants (E4$\alpha$ and E4$\omega$), as these mutants were not expected to influence the epitopes of this mAb. On the other hand, disruption of the higher affinity E4$\alpha$ epitope abolishes binding of Ki-1 while the variation in the low-affinity E4$\omega$ epitope showed no effect on binding. These findings can be explained by the epitope mapping results which demonstrated that epitope E4$\omega$ yielded a weaker Ki-1 signal than epitope E4$\alpha$, indicating a lower contribution of binding energy of the E4$\omega$ epitope. Thus, mutations in this part of the discontinuous epitope should have less influence on the overall binding of Ki-1. A loss of binding activity through mutations in E4$\omega$ is not seen in the western blot, since this is only a qualitative test. On the other hand Ki-1 must recognize a discontinuous epitope due to the clear difference in binding acitivity between CD30wt and the two epitope mutants.

### Affinity measurements

We attempted to verify the Ki-1 epitope analysis also by affinity measurements using a BIAcore instrument and chip-immobilized Ki-1. The signals obtained after binding the peptides representing the Ki-1 epitopes to the mAb ranged from 7 to 40 RU (peptide 1) and 10 to 31 RU (peptide 2). The calculated $K_D$ values were $2.6 \times 10^{-4}$ and $7.9 \times 10^{-5}$ M, respectively, showing that both peptides display significant and similar affinities to Ki-1. However, the dissociation constant of soluble CD30[ex] could not be exactly determined, most likely due to some precipitation of CD30 on the sensor chip. Nevertheless, the data clearly demonstrated that CD30 bound Ki-1 with considerably higher affinity ($<10^{-7}$ M) than any of the peptides on their own.

The BIAcore data support our previous conclusion that the Ki-1 epitope is discontinuous, as only the combination of two separated peptides into a single discontinuous epitope can lead to such synergy effects resulting in the affinities determined. Only with both partial epitopes present does the epitope/paratope complementarity become maximal, giving rise to increased affinity/avidity. Furthermore, these results exclude the possibility that two antibody paratopes interact with two different epitopes on CD30. First, as Ki-1 consists of only one type of heavy and light chain, respectively, there is only one type of antigen binding site present. Besides, it does not seem plausible to allow for the simultaneous binding of two different epitopes to identical paratopes. Second, the spot intensifications visible for both peptides studied, clearly demonstrate the presence of a discontinuous epitope.

### Homology modeling of CD30[ex]

Antibody epitopes are usually found on the surface of proteins and most often in loop regions where induced fit rearrangements of the binding partners are more feasible. To support our experimental results, homology modeling of CD30[ex] was performed. The ectodomains of proteins of the TNFR superfamily consist of cysteine-rich domains (CRDs) (Naismith and Sprang, 1998; Locksley *et al.*, 2001). CD30 belongs to this superfamily as well, but in contrast to all other members, its extracellular part is significantly longer. Using sequence alignments, the typical CRDs and the conserved Cys-bridging pattern could be identified in CD30[ex] (data not shown). Based on these conserved features and the crystal structure of TNFR1, a homology model for the N-terminal domain of CD30 was generated.

As expected, the model [Plate 1(B)] showed many structural similarities to TNFR1 with most of the disulfide bridges located at conserved positions. Using the definitions of Naismith and Sprang (1998), the d1-domain can be divided into three cysteine-rich repeats (CRD1 to CRD3), each of which contains two characteristic modules based on the cysteine-bridging pattern. The CRD3 subdomain of the modeled CD30-d1 [marked by a circle in Plate 1(B)] is the only region that shows significant folding differences to TNFR1. In particular, two loops show different conformations due to altered disulfide bridge positions when compared to TNFR1. Interestingly, CRD3 in CD30 reveals higher similarity to TNFR2 than to TNFR1 at the sequence level (data not shown). The latter is built by the two modules A1-B2, whereas CD30 and TNFR2 show an A2-B1 organization (Naismith and Sprang, 1998; Bodmer *et al.*, 2002).

The Ber-H2 (E1) and Ki-1 (E4$\alpha$) epitopes as well as the Ki-2 (E2) and R4-4 (E3) binding regions could be located in flexible loops on the surface of the molecule which appear easily accessible for antibodies. These results are an additional indication for the validity of the experimental data. The linker region and the second external domain of CD30[ex] could not be modeled due to the lack of a suitable target structure.

## DISCUSSION

The analysis of epitopes on the human CD30 molecule which we have carried out reveals that the different epitopes are all distinct, although partly overlapping:

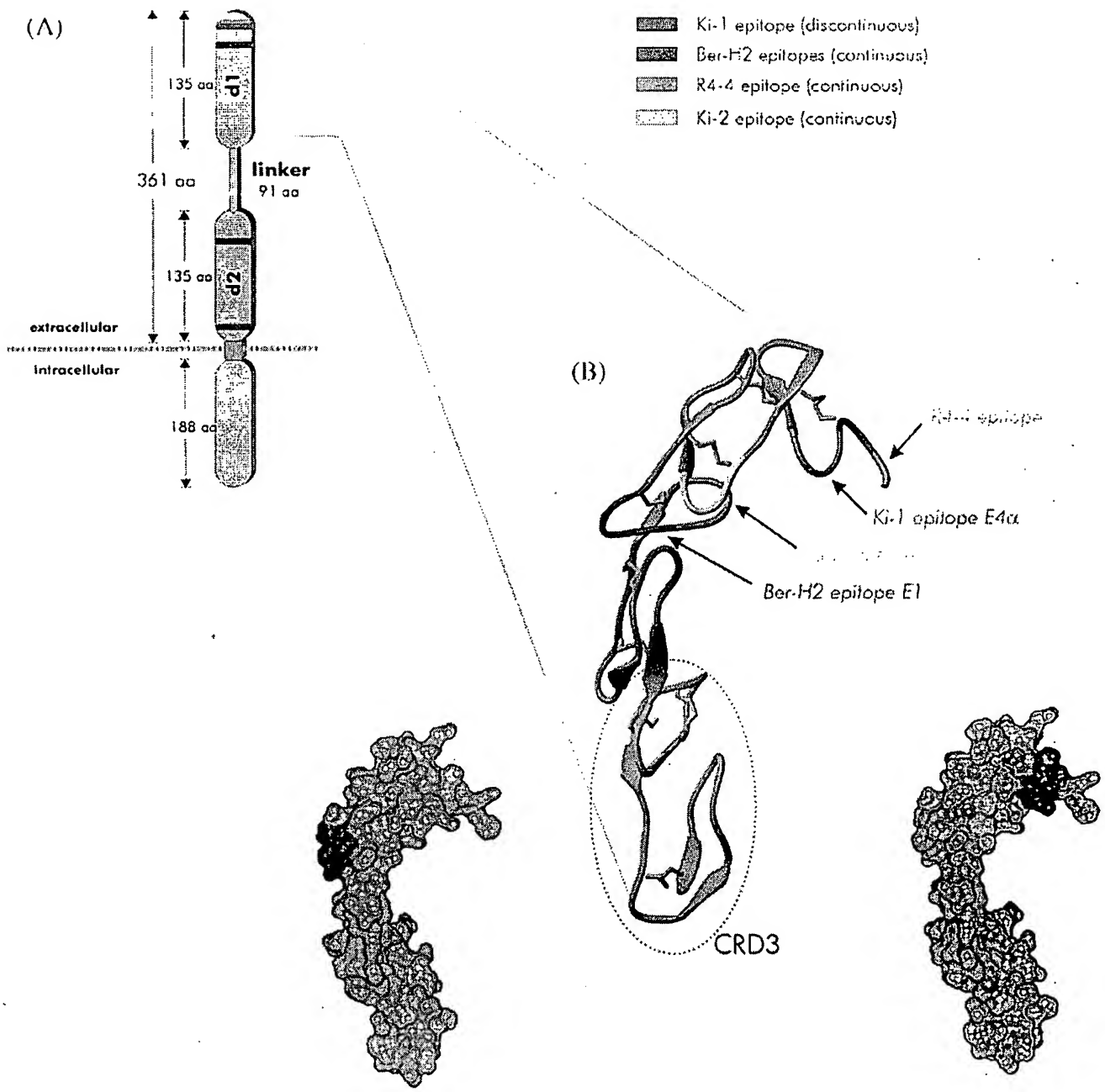


**Plate 1.** Delineation of antibody epitopes. (A) The schematic domain organization of CD30 shows the location of all antibody epitopes identified in this study. The epitopes are indicated as colored stripes. (B) The structure model of CD30-d1 is shown as ribbon-sketch. $\beta$-Strands are indicated as arrows, disulfide bridges in green. Antibody epitopes are color-coded as indicated. The epitope of the scFv R4-4 is only partly represented, as 18 N-terminal residues are missing in the model structure. CRD3, where the largest differences to the target TNFR1 are evident, is marked with a circle (see text). The Connolly representations below visualize the localization of the respective epitope on the surface of the CD30 molecule. Identical color-coding was used for all subfigures as indicated.

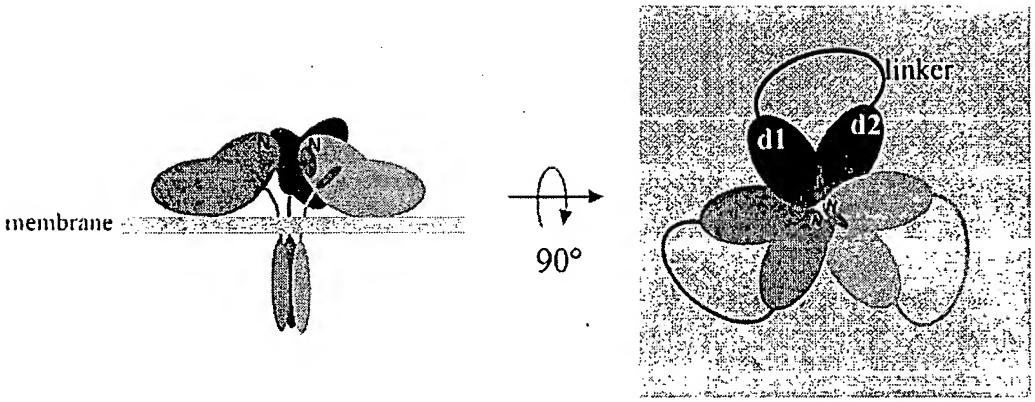


**Plate 2.** Topological model of the CD30 trimer. The arrangement of the monomers and domains [see Plate 1] is based on epitope mapping and biochemical data (see text). The discontinuous Ki-1 epitope is highlighted in red.

firstly, only two 'classical', continuous determinants (of Ki-2 and scFv R4-4) were found. Secondly, the Ber-H2 epitope occurs twice on CD30[ex], possibly contributing to the excellent affinity of this reagent (unpublished results). This unusual feature is also likely to aid in the successful therapeutic application of Ber-H2, since it is most improbable that a $CD30^+$ tumor cell can mutate both epitopes in parallel to escape Ber-H2-dependent cytotoxicity. Finally, Ki-1 recognizes a discontinuous epitope composed of amino acids near the N- and the C-termini of CD30[ex]. The fact that interaction of this mAb with the peptide spots could only be detected after amplification of the reaction indicates that both peptides contribute synergistically to Ki-1 reactivity/avidity with CD30 molecules expressed on cells. The low affinity of the isolated peptides (peptide 3 and 116) in the BIAcore analysis supports this conclusion, although it is not entirely clear why CD30[ex] with a mutated E4$\omega$ epitope can still be detected by Ki-1 [see Fig. 4(B)]. Unspecific binding of antibody to peptide or cross reactivity can in principle occur, but the SPOT technique has proven to be a particularly reliable method for epitope identification. This is mainly due to the high density of peptides on the membrane, and several contact sites, linear and nonlinear, accessible and hidden in native protein complexes have been characterized (for review see Kramer and Schneider-Mergener, 1998; Kramer *et al.*, 1999). However, these results are supported also by a CD30-truncation analysis (Dürkop *et al.*, 1992), showing that the Ki-1 determinant must be located within the first 90 N-terminal amino acids. Possibly, the E4$\omega$ epitope contributes less to Ki-1 binding in intact CD30 molecules or CD30 fragments.

In connection with the epitope analysis, the homology modeling depicts several exposed regions on the CD30 molecule. In particular, the epitopes of Ki-2, R4-4, Ber-H2 (E1), and Ki-1 (E4$\alpha$) should easily be accessible for antibodies or other interacting molecules [Plate 1(B)]. On the basis of competition assays, Horn-Lohrens and coworkers were able to define three groups of mAb recognizing different regions of CD30 (Horn-Lohrens *et al.*, 1995). Cluster A was detected by Ki-2, Ki-4, Ki-6, Ki-7, HRS-1, HRS-2, and Ber-H2, while Ki-1, Ki-5 and M67 recognized cluster B. Three other reagents revealed the existence of a further cluster C. Interestingly, Ki-2 was not able to inhibit Ber-H2 binding significantly in this study, although Ber-H2 proved to be an efficient inhibitor of Ki-2 binding. A probable explanation for this result may be the fact that Ber-H2 binds to two distinct epitopes (E1 and E1′), while Ki-2 detects only a single determinant.

It has been reported that the binding of a CD153/CD8$\alpha$ fusion protein to CD30 can be blocked by preincubation with mAb detecting clusters A and C (Franke *et al.*, 2000). Reagents detecting cluster B determinants (like Ki-1) appear not to interfere with the binding of the fusion protein. Therefore, the Ber-H2 (E1) and the Ki-2 epitopes are likely to be close to the residues contacted by CD153. This, however, does not seem to be the case for the amino acids making up the Ki-1 epitopes (Franke *et al.*, 2000) which is remarkable, since, like CD153, Ki-1 has been reported to trigger CD30 signaling (Grell *et al.*, 1999). This conclusion has also been reached by Franke *et al.* (2000) on the basis of their competition studies. It should be kept in mind, however, that a comparatively large antibody molecule could possibly prevent CD153 binding even when the respective binding sites on CD30 are not in close vicinity. Nevertheless, our epitope analyses together with the results obtained previously (Horn-Lohrens *et al.*, 1995; Franke *et al.*, 2000) suggest that CD153 interacts with CD30 in the first third (CRD1) of the N-terminal domain [CD30[ex]-d1, Plate 1(B)].

Possibly, the most interesting implication of our results emerges from the definition of the Ki-1 epitopes: since E4$\alpha$ and E4$\omega$ are simultaneously bound by the Ki-1 paratope, they must be in immediate proximity on the CD30 molecule. Therefore, the N-terminal region carrying E4$\alpha$ is expected to be close to the cell membrane where E4$\omega$ must locate, so that CD30 'bends back' onto itself, making it very unlikely that it occurs in an extended conformation on the membrane. Instead, we suggest a 'flower-like' structure for the CD30 homotrimer [Plate 2]. In this model, the N-termini of the three molecules are pointing towards each other, allowing the interaction of the three PLADs. This is a prerequisite for homotrimerization of proteins belonging to the TNFR superfamily (Chan *et al.*, 2000; Locksley *et al.*, 2001). PLAD interaction would probably not be favored if the three CD30 molecules within the trimer would contact each other only via their linker regions, since this would most likely lead to diminished contacts of the N-terminal domains (not shown). Whether all the antibody epitopes can be recognized in this trimeric state is difficult to predict, as no structure model (experimental or theoretical) of the complete molecule or the CD30-d1 domain is available. However, an arrangement of the domains where all the epitopes are accessible is conceivable. It has been argued that an optimal contact distance for two cells bearing interacting receptors of the immunoglobulin superfamily in an adhesion patch is about 140 Å (Wang *et al.*, 1999). If membrane-bound CD30 would exist in an extended form, the distance between N-terminus and the membrane might preclude interaction of a CD153-bearing cell at the optimal distance. Therefore, we favour a more condensed structure for cell membrane-expressed CD30 molecules. In support of this suggestion, mouse CD30 lacks the C-terminal external domain of human CD30, but is perfectly functional despite this deletion (Siegmund *et al.*, 2000). Possibly, membrane changes during cell–cell interactions (Qi *et al.*, 2000) might also contribute to the provision of an optimal environment favouring interaction of CD30 and CD153. Further investigations are necessary to validate the proposed structural model for CD30.

In conclusion, our study demonstrates that the delineation of epitopes on cell surface molecules is not only valuable for the understanding of antibody–target interactions. In the case reported here, it also leads to unexpected structural insights.

## Acknowledgments

We thank Dr S. Hemmann, Berlin and Dr H. Lange, Kiel, for the reagents R4-4 and Ki-2, C. Landgraf for the BIAcore analysis and Y. Kumbier for technical assistance.

## REFERENCES


Amakawa R, Hakem A, Kundig TM, Matsuyama T, Simard JJ, Timms E, Wakeham A, Mittruecker HW, Griesser H, Takimoto H, Schmits R, Shahinian A, Ohashi P, Penninger JM, Mak TW. 1996. Impaired negative selection of T cells in Hodgkin's disease antigen CD30-deficient mice. *Cell* **84**: 551–562.

Armitage RJ. 1997. CD153 Workshop Report. In *Leucocyte Typing VI*, Kishimoto T *et al.* (eds). Garland Publishing, New York; 98–100.

Bodmer JL, Schneider P, Tschopp J. 2002. The molecular architecture of the TNF superfamily. *Trends Biochem. Sci.* **27**: 19–26.

Chan FK, Chun HJ, Zheng L, Siegel RM, Bui KL, Lenardo MJ. 2000. A domain in TNF receptors that mediates ligand-independent receptor assembly and signaling. *Science* **288**: 2351–2354.

Dürkop H, Latza U, Hummel M, Eitelbach F, Seed B, Stein H. 1992. Molecular cloning and expression of a new member of the nerve growth factor receptor family that is characteristic for Hodgkin's disease. *Cell* **68**: 421–427.

Dürkop H, Oberbarnscheidt M, Latza U, Bulfone-Paus S, Hirsch B, Pohl T, Krause H, Hummel M, Stein H. 2000. The restricted expression pattern of the Hodgkin's lymphoma-associated cytokine receptor CD30 is regulated by a minimal promoter. *J. Pathol.* **192**: 182–193.

Falini B, Bolognesi A, Flenghi L, Tazzari PL, Broe MK, Stein H, Dürkop H, Aversa F, Corneli P, Pizzolo G. 1992. Response of refractory Hodgkin's disease to monoclonal anti-CD30 immunotoxin. *Lancet* **339**: 1195–1196.

Frank R. 1992. Spot synthesis: an easy technique for the positionally addressable, parallel chemical synthesis on a membrane support. *Tetrahedron* **48**: 9217–9232.

Franke AC, Jung D, Ellis TM. 2000. Characterization of the CD30L binding domain on the human CD30 molecule using anti-CD30 antibodies. *Hybridoma* **19**: 43–48.

Grell M, Zimmermann G, Gottfried E, Chen CM, Grunwald U, Huang DC, Lee YHW, Dürkop H, Engelmann H, Scheurich P, Wajant H, Strasser A. 1999. Induction of cell death by tumour necrosis factor (TNF) receptor 2, CD40 and CD30: a role for TNF-R1 activation by endogenous membrane-anchored TNF. *EMBO J.* **18**: 3034–3043.

Hartmann F, Renner C, Jung W, Deisting C, Juwana M, Eichentopf B, Kloft M, Pfreundschuh M. 1997. Treatment of refractory Hodgkin's disease with an anti-CD16/CD30 bispecific antibody. *Blood* **89**: 2042–2047.

Hemmann S, Ziegler A, Uchanska-Ziegler B. 2002. Expression of the cystein-rich extracellular domain of CD30 in Drosophila melanogaster S2 cells and selection of CD30-specific human single-chain Fv antibodies from a phage display library. In *Leucocyte Typing VII*, Mason D *et al.* (eds). Oxford University Press, New York; 463–466.

Horie R, Watanabe T. 1998. CD30: expression and function in health and disease. *Sem. Immunol.* **10**: 457–470.

Horn-Lohrens O, Tiemann M, Lange H, Kobarg J, Hafner M, Hansen H, Sterry W, Parwaresch RM, Lemke H. 1995. Shedding of the soluble form of CD30 from the Hodgkin-analogous cell line L540 is strongly inhibited by a new CD30-specific antibody (Ki-4). *Int. J. Cancer* **60**: 539–544.

Jones TA, Kjeldgaard M. 1997. Electron-density map interpretation. *Meth. Enzymol.* **277**: 173–208.

Klussmann S, Nolte A, Bald R, Erdmann VA, Furste JP. 1996. Mirror-image RNA that binds D-adenosine. *Nat. Biotechnol.* **14**: 1112–1115.

Kramer A, Schneider-Mergener J. 1998. Synthesis and screening of peptide libraries on continuous cellulose membrane supports. *Methods Mol. Biol.* **87**: 25–39.

Kramer A, Reineke U, Dong L, Hoffmann B, Hoffmuller U, Winkler D, Volkmer-Engert R, Schneider-Mergener J. 1999. Spot synthesis: observations and optimizations. *J. Pept. Res.* **54**: 319–327.

Locksley RM, Killeen N, Lenardo MJ. 2001. The TNF and TNF receptor superfamilies: integrating mammalian biology. *Cell* **104**: 487–501.

Lotze MT, Zola H. 1997. CD30 Workshop Report. In *Leucocyte Typing VI*, Kishimoto T *et al.* (eds). Garland Publishing, New York; 489–491.

Muta H, Boise LH, Fang L, Podack ER. 2000. CD30 signals integrate expression of cytotoxic effector molecules, lymphocyte trafficking signals, and signals for proliferation and apoptosis. *J. Immunol.* **165**: 5105–5111.

Naismith JH, Sprang SR. 1998. Modularity in the TNF-receptor family. *Trends Biochem. Sci.* **23**: 74–79.

Naismith JH, Devine TQ, Kohno T, Sprang SR. 1996. Structures of the extracellular domain of the type I tumor necrosis factor receptor. *Structure* **4**: 1251–1262.

Nolte A, Klussmann S, Bald R, Erdmann VA, Furste JP. 1996. Mirror-design of L-oligonucleotide ligands binding to L-arginine. *Nat. Biotechnol.* **14**: 1116–1119.

Pallesen G, Hamilton-Dutoit SJ. 1988. Ki-1 (CD30) antigen is regularly expressed by tumor cells of embryonal carcinoma. *Am. J. Pathol.* **133**: 446–450.

Peitsch MC. 1996. ProMod and Swiss-Model: Internet-based tools for automated comparative protein modeling. *Biochem. Soc. Trans.* **24**: 274–279.

Qi SY, Groves JT, Chakraborty AK. 2000. Synaptic pattern formation during cellular recognition. *Proc. Natl Acad. Sci. USA* **98**: 6548–6553.

Reineke U, Sabat R, Welfle H, Volk HD, Schneider-Mergener J. 1999a. Synthesis of a mimic of a discontinuous binding site on interleukin-10. *Nat. Biotech.* **17**: 271–275.

Reineke U, Kramer A, Schneider-Mergener J. 1999b. Knowledge- and library-based mapping of discontinuous protein-protein-interactions by spot synthesis. *Curr. Top. Microbiol. Immunol.* **243**: 23–36.

Schwab U, Stein H, Gerdes J, Lemke H, Kirchner H, Schaadt M, Diehl V. 1982. Production of a monoclonal antibody specific for Hodgkin and Sternberg-Reed cells of Hodgkin's disease and a subset of normal lymphoid cells. *Nature* **299**: 65–67.

Schwarting R, Gerdes J, Durkop H, Falini B, Pileri S, Stein H. 1989. Ber-H2: a new anti-Ki-1 (CD30) monoclonal antibody directed at a formol-resistant epitope. *Blood* **74**: 1678–1689.

Siegmund T, Armitage N, Wicker LS, Peterson LB, Todd JA, Lyons PA. 2000. Analysis of the mouse CD30 gene: a candidate for the NOD mouse type 1 diabetes locus Idd9.2. *Diabetes* **49**: 1612–1616.

Stein H, Mason DY, Gerdes J, O'Connor N, Wainscoat J, Pallesen G, Gatter K, Falini B, Delsol G, Lemke H. 1985. The expression of the Hodgkin's disease associated antigen Ki-1 in reactive and neoplastic lymphoid tissue: evidence that Reed-Sternberg cells and histiocytic malignancies are derived from activated lymphoid cells. *Blood* **66**: 848–858.

Wang JH, Smolyar A, Tan K, Liu JH, Kim M, Sun ZY, Wagner G, Reinherz EL. 1999. Structure of a heterophilic adhesion complex between the human CD2 and CD58 (LFA-3) counter-receptors. *Cell* **97**: 791–803.